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Full Paper

Heterogeneity of gut microbiome compositions in the third decade of life in Japanese women: insights from a comparative analysis

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The reasons for sex-associated gut microbiota differences have not been determined, and although sex hormones, diet, and other factors are considered to contribute to them, many of these factors are age related. To shed light on this complex interplay, our study aimed to investigate and compare the gut microbial compositions of males and females across a broad range of ages, aiming to identify sex-associated disparities and potential causal factors. Our study encompassed a comprehensive analysis of gut microbiota data obtained from 444 Japanese individuals, ranging from newborns to centenarians, sourced from the DNA Data Bank of Japan. We categorized the subjects into 13 distinct age groups and examined their relative microbial abundances, as well as alpha and beta diversities, in relation to sex and age. No difference was observed between gut microbiota relative abundances or alpha diversities between men and women at any age. However, the study showed that the heterogeneity of gut microbiota among women in their 20s was greater than in men. To confirm the general occurrence of this difference, we conducted additional analyses using seven datasets: three from Japan and four from other countries. Interestingly, this variance was particularly noticeable within Japanese women. We also showed a potential link between the observed heterogeneity and dietary fiber intake. It is hoped this study will provide clues that aid in the identification of factors responsible for sex-associated differences in gut microbiota compositions.

Key words: human gut microbiota, sex difference, dietary fiber

INTRODUCTION

The gut microbiota is the most extensively studied human microbiome. We now know that the gut microbiota plays an important role in maintaining the physiology of the human immune system and metabolism and that microbial dysbiosis is associated with several diseases. Nevertheless, it is still unclear whether the gut microbiota compositions of men and women differ. As summarized by Kim *et al.*, several comparative studies on the gut microbiota by sex have reported differences in gut microbial compositions or alpha diversities between males and females [1]. However, the reported results are inconsistent, and other studies have reported no differences between the sexes. Studies to date suggest that sex hormones, drugs, diet, body mass index (BMI), and colon transit time influence sex-associated gut microbiota differences and that the factors responsible are age dependent. Sex hormones are a likely factor in such differences after puberty, though diet is the most potent modulator of gut microbial composition and dietary preferences vary with age [2–5]. Likewise, BMI and colon transit time are significantly

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dependent on age [6, 7]. Therefore, studies on sex differences in gut microbiota compositions throughout the life cycle should enable elucidation of the factors that contribute to these differences.

To date, only one study has investigated sex-associated differences in gut microbiota compositions by age, and that study reported no sex-associated difference in healthy individuals [8]. However, only 75 healthy individuals were included in the study, and the participants were classified into only 5 age groups. Therefore, the present study was conducted to investigate sex-associated differences in gut microbiota compositions in more than 400 healthy individuals assigned to 13 age groups.

MATERIALS AND METHODS

Data acquisition

A total of 453 samples of gut microbiomes were obtained from the DNA Data Bank of Japan (DDBJ) under accession numbers DRA004160 and DRA005774 [9, 10]. The data used were de-identified and publicly available; thus, Institutional Review Board approval was not required. They included raw 16S rRNA gene amplicon sequences obtained from stool samples of healthy volunteers in Japan, which ranged from newborns to centenarians. The details of the methods used to collect the data, from stool collection to sequencing, are described in previous publications [9, 10]. Briefly, the variable regions V3–4 of the bacterial 16S rRNA gene were amplified using the primer pair Tru357F and Tru806R. Polymerase chain reaction (PCR) products were then sequenced with pair-ends using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). The read length used was 278 nucleotides.

Data processing

Three samples (DRR049304, DRR049305, and DRR049305) were excluded from the analysis because only one end of the pair-ends reads was released. Quality filtering and processing were performed using the DADA2 package in R [11]. Forward and reverse reads were truncated at positions 265 and 210, respectively, which left a 25-bp overlap for merging. One sample (DRR049275) was excluded from the analysis because its read lengths were 233 and 228 in the forward and reverse directions, respectively. The maximum numbers of expected errors (maxEE) were set at 2 and 4 for forward and backward reads, respectively. All other parameters were set at the DADA2 default values. Filtered and trimmed reads were then dereplicated and denoised according to the DADA2 pipeline tutorial. Pair-end reads were merged to obtain longer contigs. An amplicon sequence variant (ASV) table was constructed based on these contigs. Chimeric sequences were removed from the ASV table. The numbers of reads passed through each data processing step are shown in Table 1.

Taxonomy assignment and data transformation

Taxonomic assignments were performed on the final ASV table based on DADA2-formatted reference FASTA version 138 (silva_nr_v138_train_set) [12, 13]. Subsequently, the final ASV table was converted to a phyloseq object [14]. To remove rarely found taxa among samples, taxonomic filtering was applied. Thus, five phyla (Campylobacterota, Cyanobacteria, Deferribacterota, Patescibacteria, and Synergistota) with less than four contigs across the samples were removed from the phyloseq object. Prevalence filtering was then performed to remove low-prevalence taxa and possible outliers using a prevalence threshold of 22.45, which resulted in the removal of taxa present in less than 5% of samples. The taxonomic- and prevalence-filtered phyloseq object was then agglomerated at genus rank to merge redundant species. Finally, the count data of the phyloseq object were converted to relative abundances.

Analysis of sex-associated differences in gut microbiomes by age

Samples were classified into 13 predefined age groups (Table 2). The samples were divided into 10 year age groups to compare the differences in the composition of the gut microbiota between men and women by age, except for subjects under 10 years of age; they were classified into 4 groups according to their diet stage. All five subjects over 100 years old were women and thus were excluded. The data of the 444 remaining subjects were subjected to analysis. To compare gut microbial compositions by sex and age, the 20 taxa with the highest prevalences from the 444 samples were selected. The average relative abundances of these 20 taxa were plotted by age group according to sex. Principal coordinate analyses (PCoAs) for the relative abundances of the

Table 1. Overview of total read counts

Group	Input	Filtered	DenoisedF	DenoisedR	Merged	Nonchim
1	10,303	4,661	4,605	4,594	4,425	4,402
2	8,846	3,299	3,247	3,220	3,054	3,019
3	20,476	10,391	10,253	10,258	9,766	8,852
4	10,635	4,647	4,552	4,497	4,106	4,103
5	11,140	4,944	4,855	4,790	4,296	4,289
6	16,899	10,575	10,338	10,372	9,520	8,150
7	13,762	7,276	7,087	7,068	6,389	6,010
8	12,901	5,924	5,766	5,733	5,153	4,861
9	19,033	11,576	11,224	11,345	10,286	9,033
10	12,715	6,931	6,571	6,688	5,765	5,397
11	14,763	8,213	7,717	7,923	6,738	6,246
12	14,396	6,849	6,612	6,553	5,781	5,663
13	13,761	6,151	5,910	5,751	5,090	5,077
14	18,579	8,524	8,350	8,252	7,677	7,531

The average number of reads per sample at each filtering step is provided.

Table 2. Sample distributions and age groupings

Group	Segmentation	Mean	SD	Number of samples	F	M
1	Preweaning [0–0.3]	0.3	0.1	10	5	5
2	Weaning [0.4–1]	0.7	0.2	11	3	8
3	Weaned [1–3]	2.4	0.6	23	12	11
4	4–9 years old	5.9	1.8	17	10	7
5	10–19 years old	14.1	3.6	10	3	7
6	20–29 years old	25.7	2.7	42	28	14
7	30–39 years old	34.4	2.6	117	61	56
8	40–49 years old	43.4	3.1	37	23	14
9	50–59 years old	53.4	2.5	34	20	14
10	60–69 years old	64.2	2.9	42	28	14
11	70–79 years old	75.5	2.9	31	19	12
12	80–89 years old	83.2	2.4	51	34	17
13	90–99 years old	94.2	2.7	19	15	4
14	≥100 years old	101.6	1.8	5	5	0

SD: standard deviation; F: female; M: male.

top 20 prevalent taxa or sample dispersions were performed using the Bray-Curtis dissimilarity and unweighted and weighted UniFrac distances.

Validation analysis

We conducted a validation analysis using publicly available datasets from external sources. As illustrated in the flowchart in Supplementary Fig. 1, a total of seven distinct datasets, each comprising healthy individuals aged between 20 and 29, were chosen (Supplementary Table 1). Specifically, JP2 (Japan), the UK (United Kingdom), and the US (United States) were selected from the GMrepo repository [15]. Two additional Japanese cohorts (JP3 and JP4) were included from the previous published literature [16]. Raw FASTQ sequences were obtained from DDBJ with the identifiers listed in Supplementary Table 2. Furthermore, OTU (operational taxonomic unit) tables from the studies in China (CN) and Colombia (CO) were acquired from the Qiita database [17]. A comprehensive overview of these additional datasets is available in Supplementary Tables 1 and 2. To ensure the methodological consistency, we employed identical analytical methods as detailed above to scrutinize these diverse datasets.

Dietary intake analysis

For assessment of dietary intake across diverse age groups and genders, we employed a distinct dataset sourced from the 2016 Japan National Health and Nutrition Survey (NHNS) [18]. Coefficients of variation of nutrient intakes were calculated by dividing the standard deviations of nutrient intakes by their means.

Statistical analysis

To calculate UniFrac distances between samples, a phylogenetic tree was built based on the similarity of the inferred sequence variants obtained from DADA2. Microbial diversity within samples (alpha diversity) of males and females was compared using Shannon's and Simpson's diversity indices. Alpha diversities were calculated using ASV counts without taxonomic and prevalence filtering. Permutational analysis of variance (PERMANOVA) was performed to explore differences between gut microbiota compositions by sex. Analysis of group dispersion homogeneities by sex and specific taxa was also performed using permutational multivariate analysis of dispersion (PERMDISP) and Levene's test. The level of statistical significance was set at $p<0.05$. Statistical analysis and visualization were performed using R.

RESULTS

The average relative abundances of the gut microbiota at the phylum and family level by sex and age group are shown in Fig. 1. Overall, phylum Bacillota and family *Lachnospiraceae* were dominant across all age groups in both sexes, except the first two age groups (<1 year of age). In addition, a decrease in the phylum Actinomycetota and increase in the phylum Bacillota were observed as age increased. However, there were no discernible sex differences in microbiota composition across all age groups. Also, analysis at the family taxonomic level did not identify any noticeable differences in gut microbiota composition by sex in any age group.

PCoA was performed to explore the effects of sex on gut microbiota composition (Fig. 2A) and revealed differences in gut microbiota composition by age but not by sex. Alpha diversity was also observed to increase with age, as has been previously reported, but to be unaffected by sex (Fig. 2B) [9]. The 95% confidence intervals of alpha diversity trends by age for males and females nearly overlapped.

PCoA plots of age groups showing statistically significant sex-associated differences by PERMANOVA are shown in Fig. 3. PERMANOVA based on Bray–Curtis dissimilarities and unweighted UniFrac distances revealed significant sex differences in all subjects (0–99 years old) and age group 7 (30–39 years old). However, PCoA plots for these two age groups (age groups ALL and 7 in Fig. 3) showed that the ordination positions and distributions of males and females were similar. Moreover, R^2 values were very low, ranging from 0.004 to 0.021.

Also, the beta diversities of the gut microbiota in females and males in each age group by the PERMDISP method are shown in Fig. 4. Significant statistical heterogeneity of beta diversity was observed between males and females in age groups 6 (20–29 years old) and 13 (90–99 years old). The PCoA plots of these

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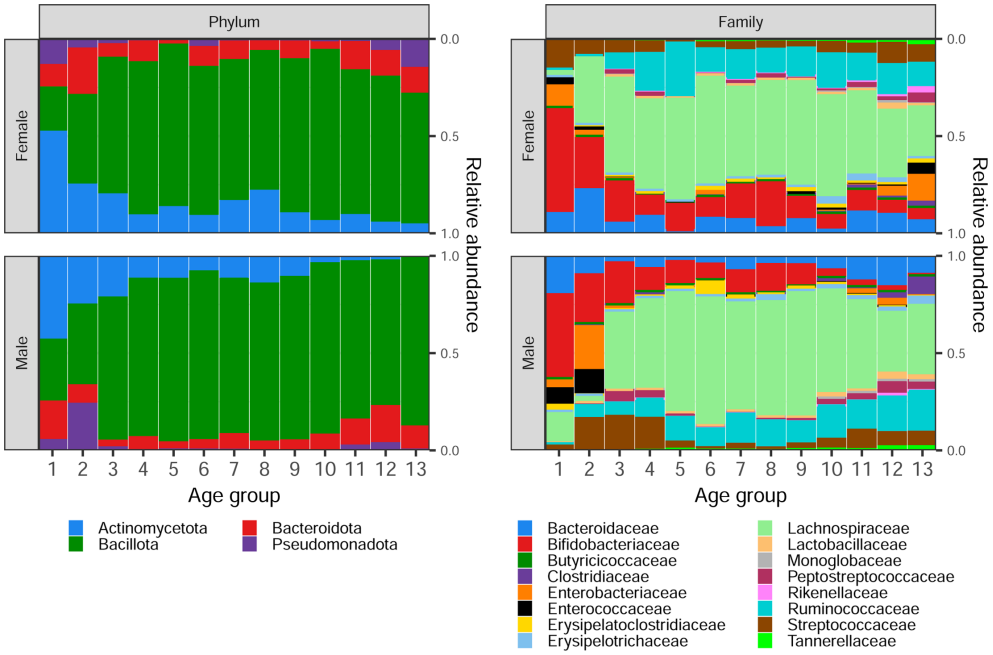

Fig. 1. Analysis of sex-associated gut microbiome differences by age: relative abundances of the gut microbiota.
Different colors represent different bacteria at the phylum and family levels.

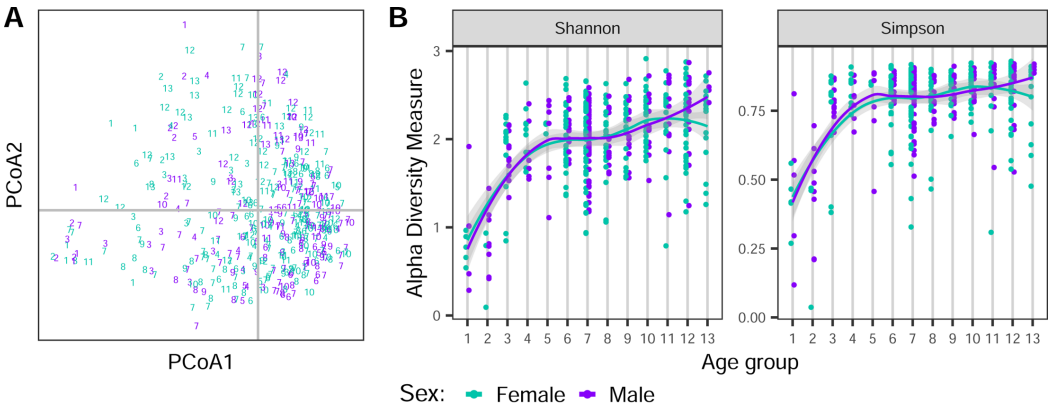

Fig. 2. Analysis of sex-associated gut microbiome differences by age.
(A) Principal coordinate analysis. (B) Alpha diversity analysis findings. The gray areas are 95% confidence intervals. Green and purple indicate females and males, respectively. PCoA: Principal coordinate analyses.

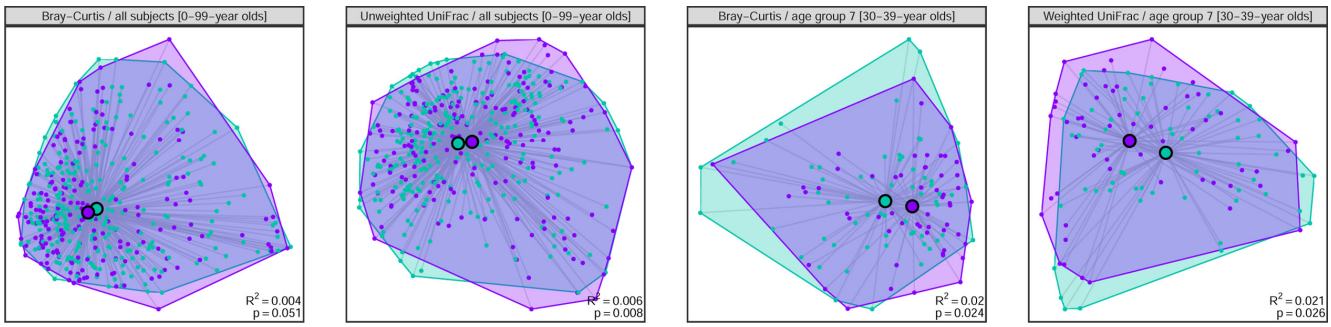

Fig. 3. Statistical analysis of sex-associated gut microbiome differences by age using Bray-Curtis dissimilarity and unweighted/weighted UniFrac distances.

Permutational analysis of variance was performed to explore differences in gut microbiota compositions. Green indicates females; purple indicates males. Circles indicate centroids of each group dispersion.

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two age groups are shown in Fig. 5. Females in age groups 6 and 13 showed greater within-group dispersions of beta diversity than males (p-values of 0.004 and 0.013, respectively). The F-statistics for the analysis of homogeneity of group dispersions in these two age groups ranged from 7.573 to 8.828. Thus, the heterogeneity of gut microbiota compositions among women was found to be greater than among men in these two age groups (Fig. 5).

To further validate the observed gender difference among individuals in their 20s, we extended our analysis to additional datasets that each comprised healthy individuals aged between 20 and 29. This encompassed three Japanese cohorts (JP2, JP3, and JP4) and those for four other nationalities (CN, UK, CO, and US). Interestingly, a tendency towards greater heterogeneity among women compared with men was discernible, particularly within the Japanese population (Fig. 6 and Supplementary Fig. 2).

The relative abundances of the gut microbiota at the phylum level by sex in age group 6 are shown in Fig. 7. The females of this group showed a decrease in Bacillota abundance and an increase in Bacteroidota and Actinomycetota abundance. On the other hand, the males of age group 6 had homogeneous gut microbiome compositions, with Bacillota being dominant. Levene's test was performed to assess the equality of dispersion

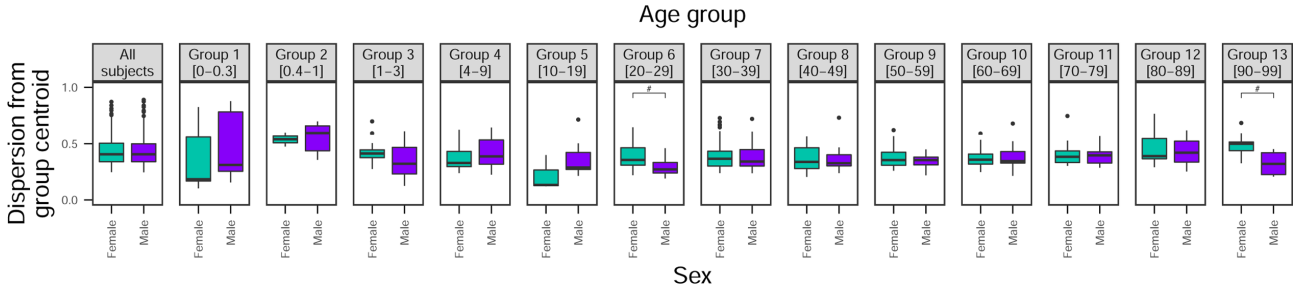

Fig. 4. Statistical analysis of sex-associated gut microbiome differences by age using Bray–Curtis dissimilarity: boxplot of the group dispersions.

The numbers in brackets indicate the age ranges. Green indicates females; purple indicates males. #Statistically significant.

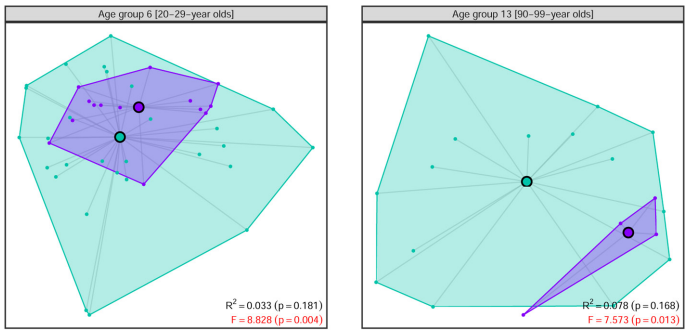

Fig. 5. Statistical analysis of sex-associated gut microbiome differences by age using Bray–Curtis dissimilarity.

Permutational multivariate analysis of the dispersion method was conducted to analyze the homogeneity of group dispersions in age groups 6 and 13. Green indicates females; purple indicates males. Circles indicate the centroids of each group dispersion.

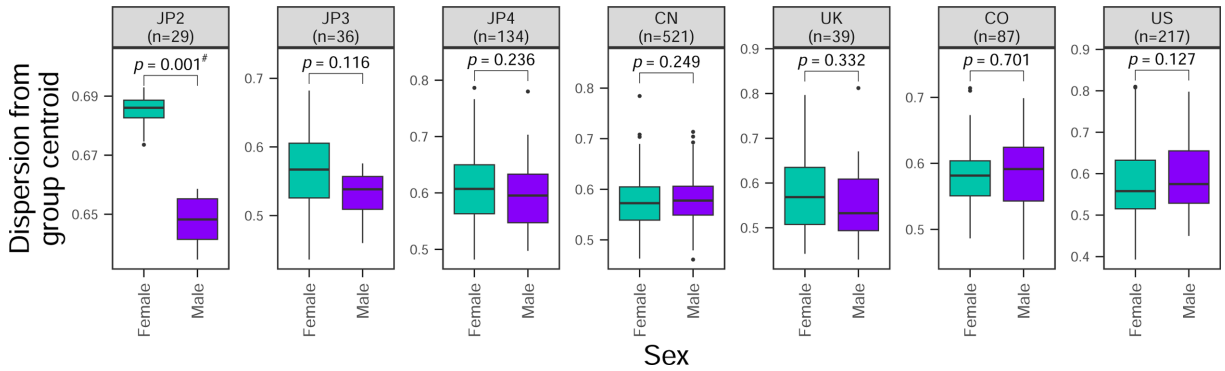

Fig. 6. Dispersion of gut microbiota among individuals aged 20 to 29 from different nationalities, categorized by sex.

The numbers in parentheses denote the number of cases. Green indicates females; purple indicates males. #Statistically significant. JP: Japan; CN: China; UK: United Kingdom; CO: Colombia; US: United States.

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for four phyla (Bacteroidota, Bacillota, Actinomycetota, and Pseudomonadota) by sex in age group 6. As shown in Table 3, the dispersion of Bacillota abundance was significantly different in women compared with men in age group 6 (p-value=0.031). However, this significant heterogeneity of Bacillota in women was not consistently observed in other datasets (Supplementary Fig. 3).

To uncover the drivers contributing to the observed heterogeneity within age group 6, we employed a distinct dataset from the NHNS to investigate differences in dietary intake between women and men. Coefficients of variation of nutrient intakes by age and sex in the general Japanese population are shown in Fig. 8A. Women in their 20s had the largest level of dispersion for dietary fiber intake. Out of 444 study subjects, 86 subjects from 22 families were included. These 86 subjects showed more homogenous intestinal microbiome compositions than the other 358 subjects who were not members of their families (Fig. 8B).

DISCUSSION

It is well known that the gut flora plays an important role in maintaining the normal functions of the human body [19, 20]. In addition, changes in the intestinal microbial community are known to be associated with not only gastrointestinal diseases but also extraintestinal diseases such as tumors, metabolic diseases, allergies, and immune disorders [1, 21, 22]. Therefore, it is important to understand what constitutes a normal gut microbial composition. However, we do not yet know the difference between

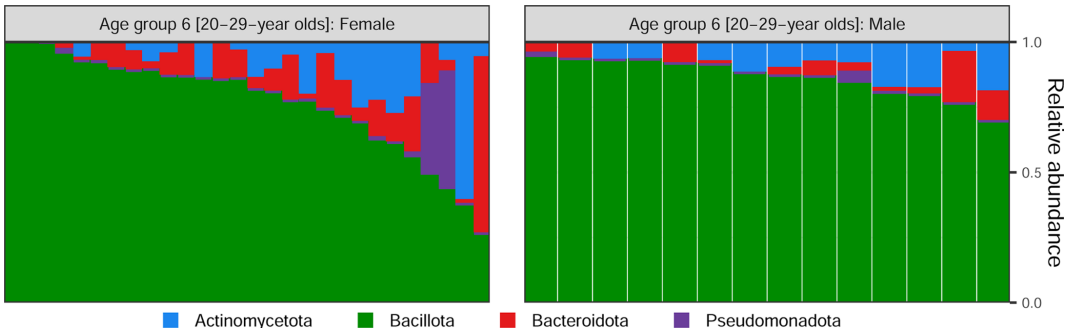

Fig. 7. Analysis of sex-associated microbial compositions in age group 6 at the phylum level.
Relative abundance was used to represent the gut microbial composition in each group. Different colors indicate different bacteria at the phylum level.

Table 3. Analysis of different dispersions in phylum abundance by sex of age group 6 (20–29 years old) using Levene's test

	Female			Male			p-value
	Mean	SD	CV	Mean	SD	CV	(Levene's test)
Actinomycetota	0.1003	0.1324	132.01	0.0833	0.0644	77.29	0.2381
Bacillota	0.7683	0.1950	25.380	0.8651	0.0751	8.69	0.0310
Bacteroidota	0.1010	0.1290	127.76	0.0475	0.0542	114.01	0.2108
Pseudomonadota	0.0304	0.1038	341.49	0.0041	0.0121	294.39	0.3536

SD: standard deviation; CV: coefficient of variation.

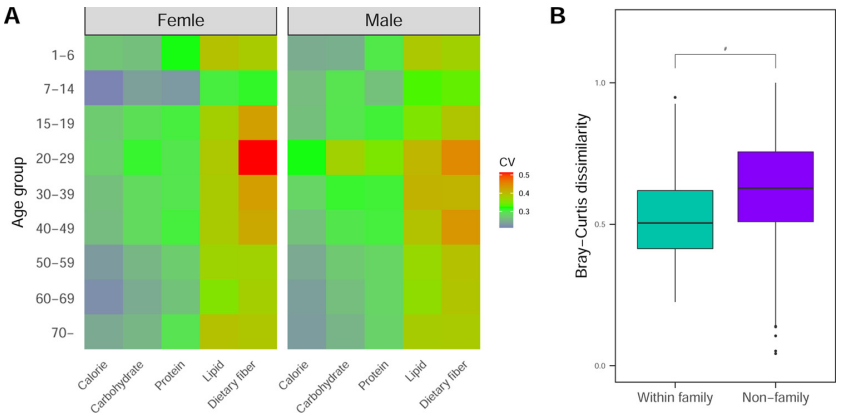

Fig. 8. Analysis of potential heterogeneity factors for intestinal microbial composition.
(A) Dietary intake analysis, derived from the Japan National Health and Nutrition Survey. (B) Comparison of group dispersions in the gut microbiome between family members and non-family members. #Statistically significant. CV: coefficient of variation.

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what constitutes a normal gut microbiota in men and women. Several studies have reported no sex differences in terms of the relative abundances or alpha diversities of the gut microbiota, but this remains an open issue [8, 23]. A study on 230 subjects aged 20 to 50 in France, Germany, Italy, and Sweden found that the *Bacteroides-Prevotella* group was richer in men than women [24]. Another study of 82 Americans aged 30 to 83 also reported a higher prevalence of *Bacteroidetes* in men [25]. In addition, the Human Microbiome Project reported that community type D, which has fewer *Bacteroides* and more *Prevotella*, is more prevalent in men [26]. However, unlike these previous studies, which found differences in the prevalences of gut microbiota in men and women, no such differences were observed across any age groups in the present study (Fig. 1). PCoA of the relative abundances of highly prevalent taxa revealed changes in gut microbial composition by age (Fig. 2), as has been previously reported, but no changes by sex [9]. Therefore, the subjects analyzed in this study exhibited few or no differences in the relative abundances of gut microbiota by sex, and even when sex appeared to affect relative abundances, differences were minor as compared with the effects of age. Chinese and Dutch studies on 551 and 1,135 subjects, respectively, reported that females exhibit higher alpha diversity than males [27, 28], but a sex-associated difference in alpha diversity was not observed in the current study in any age group (Fig. 2). Although PERMANOVA showed a significant difference in gut microbiota compositions between males and females in all subjects (0–99 years old) and age group 7 (30–39 years old), it is not possible to conclude that these differences constituted a meaningful sex-associated difference, because the R^2 values in both groups were almost zero. In addition, no significant sex-associated differences between the ordination positions or distributions were observed by PERMANOVA (Fig. 3). Accordingly, these results support the conclusion that gender does not influence the relative abundance or alpha diversity of gut microbiota. Age group 7 was the group with the most subjects (Table 2). As mentioned in a review paper by Kim *et al.* [1], studies that have reported significant sex differences in gut microbiota have been conducted on larger cohorts. Therefore, the following two interpretations were derived based on the PERMANOVA results. First, although the effect size of sex on gut microbiota compositional differences is small, the sample size in age group 7 was sufficient to detect its effect. Second, when using PERMANOVA, a more stringent p-value cutoff should have been used.

This study analyzed differences in beta diversity between men and women by age for the first time (Fig. 4). It found that intragroup dispersions (variances) among women were greater than those among men in age groups 6 and 13 (20–29 years old and 90–99 years old, respectively; Fig. 5). Although sex was not found to influence mean gut microbiota composition, sex was found to affect intragroup variance. For subjects in their 20s, differences in gut microbiota compositions among women were significantly greater than among men. Validation of this observation across the additional datasets indicates that the notable heterogeneity seen among females in age group 6 appears to be distinctive to the Japanese cohort (Fig. 6). However, it is important to interpret these findings cautiously. Despite a discernible trend indicating greater gut microbiota heterogeneity among Japanese women in their 20s, it is noteworthy that only 2 out of 4 Japanese studies exhibited statistical differences. Additional factors, such as modest samples size in some datasets, variations in experimental settings, and unequal sample sizes between sexes in certain datasets, should also be considered. Furthermore, the scarcity of previous studies encompassing a sufficient number of cases for healthy individuals aged 20 to 29 poses a challenge. A considerable number of studies lack age or sex information within their metadata, rendering future analyses unfeasible. Therefore, there arises a critical need for future investigations employing consistent research methodologies to robustly validate this hypothesis.

It is unlikely that the sex-associated difference observed in 20–29 year-olds was due to sex hormones, because were this the case, a difference should have been observed throughout the adolescent to middle-aged period. Thus, it can be deduced that factors such as diet were probably responsible for this difference in age group 6.

Diet is known to be the most potent modulator of gut microbiota composition [29]. Thus, to explore the effect of diet on gut microbiota homogeneity in men and women in their 20s, this study analyzed nutritional intake data by sex and age. In order to match subjects in the nutritional intake study and gut microbiome analysis, this study used Japanese nutritional intake information for 2016 from the NHNS. It is noteworthy that the Japanese diet is highly homogeneous throughout Japan [30]. Analysis of the effects of sex, age, and nutrient type showed that dietary fiber intake by women in their 20s was the largest difference between individuals (Fig. 8A). Furthermore, it has also been well established that high dietary fiber intake increases the diversity of gut microflora, particularly the abundances of Bacillota and Bacteroidota, two beneficial phyla of the healthy adult intestinal microbiota [4, 5, 31]. Interestingly, an analysis of changes in the relative abundances of specific taxa suggested that the alteration in dominance within the Bacillota phylum could potentially contribute to the greater heterogeneity observed among women in age group 6. This discrepancy was further emphasized by a several-fold higher coefficient of variation in females (Fig. 7 and Table 3). Of the 444 subjects, 86 belonged to 22 families. Since family members typically eat the same meals, we compared the heterogeneity of the intestinal microbiomes in these subjects and the other 358 subjects who were not members of their families (Fig. 8B). The subjects belonging to the families showed more homogenous microbial compositions than those who did not. These results suggest that a large difference in dietary fiber intake among women in their 20s led to greater intragroup heterogeneity than among men. Within-sex gut microbiota heterogeneity was also greater for females in group 13 (90–99 years old), but this difference was tenuous because there were only four males in the group.

While this research provides insights into gender-based disparities in gut microbiota composition in the third decade of life among the Japanese population, it is important to recognize that inherent variations in cohort designs and methodologies among these publicly available datasets may introduce a certain level of variability that could potentially impact the results [31]. Additionally, this study proposes a potential role for dietary fiber intake in the observed dispersion of gut microbiota among women. Nevertheless, it is important to note that the dietary data was collected from different individuals compared with the samples used in the metagenomic study. Consequently, the present study does not offer direct evidence for the underlying

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causes of sex-associated differences in gut microbiota. Therefore, this study suggests that a controlled study should be undertaken on the relationship between sex differences in gut microbiota and dietary habits. There are several studies reporting that BMI is related to the composition of the gut microbiota [8, 27, 32]. However, since the BMIs of the subjects were not known in this study, the effect of BMI on the sex-associated differences in gut microbiota identified in this study could not be evaluated.

In summary, this study affirms that the relative abundances and alpha diversity of the gut microbiota in the Japanese population are not dependent on sex, which is in line with the majority of previous studies. However, it also shows that the interindividual variability in the gut microbiota was higher among Japanese women in their 20s than in men of the same age.

INSTITUTIONAL REVIEW BOARD STATEMENT

The data used were de-identified and publicly available; thus, Institutional Review Board approval was not required.

FUNDING

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DATA AVAILABILITY STATEMENT

The data presented in this study are openly available in DNA Data Bank of Japan (DDBJ) under accession numbers DRA004160 and DRA005774. The data used in the additional analyses for other nationalities are also available as described in the Supplementary Table 2.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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